Avian Reovirus Antibody Assay by Indirect Immunofluorescence Using Plastic Microculture Plates

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ABSTRACT

An indirect fluorescent antibody test was developed to detect serum antibody to avian reovirus strain WVU2937. This test employed small multiple well plastic plates (8 x 5.5 cm) which readily fitted into the standard mechanical stage mechanism of an incident light fluorescence microscope. The small wells of the plates required minimal (10 μL) volumes of reagents. In tests on 18 sera in which the indirect fluorescent antibody, agar gel precipitin and plaque reduction methods were compared sera which gave negative results in the agar gel precipitin test were sometimes positive in the indirect fluorescent antibody and plaque reduction test, but indirect fluorescent antibody titers were lower than plaque reduction test titers. No false positive reactions were detected in 46 sera from uninoculated specific pathogen free chicks of up to eight weeks of age.

RÉSUMÉ

Cette expérience visait à développer une épreuve d'immunofluorescence indirecte, destinée à détecter les anticorps sériques à l'endroit de la souche WVU 2937 du réovirus aviaire. L'épreuve impliquait l'utilisation de plaques de plastique qui mesuraient 8 x 5,5 cm et qui étaient pourvues de plusieurs petits puits; ces plaques s'ajustèrent facilement dans le mécanisme standard du tablier d'un microscope à fluorescence, à lumière incidente. Les petits puits des plaques ne requéraient qu'une quantité infime (10 μL) de réactifs. Une étude comparative de 18 échantillons de sérum par les épreuves d'immunofluorescence indirecte, d'immunodiffusion sur gélose et de réduction des plages, révéla que les échantillons qui donnaient un résultat négatif par l'épreuve d'immunodiffusion sur gélose, se révélaient parfois positifs, avec les deux autres méthodes; dans ces cas, les titres d'anticorps décelés par l'épreuve d'immunofluorescence indirecte s'avérèrent cependant inférieurs à ceux que détectait la méthode de réduction des plages. On n'enregistra aucune réaction positive erronée, lors de l'épreuve de 46 échantillons de sérum provenant de poulets exempts d'agents pathogènes spécifiques et âgés de moins de huit semaines.

INTRODUCTION

Pursell and Cole (7) first described a method for the acetone fixation of cell monolayer cultures in plastic plates which prevented the etching of plastic by acetone. The following work was conducted to develop a technique for the assay of antibody by the indirect fluorescent antibody (IFA) method using tissue cultures grown on small plastic plates of a size which would easily fit into the slide holder of the standard mechanical stage mechanism of a fluorescence microscope. An avian reovirus, strain WVU2937, and its homologous antibody was used as a model in this study.

MATERIALS AND METHODS

CELL MONOLAYER CULTURES

African green monkey kidney cell line monolayer cultures (Vero cells)[1] were grown in Eagle's minimal essential medium with Earle's base (EMEM)[2] containing antibiotics (penicillin 100 i.u. streptomycin 100 μg and mycostatin 25 units, per mL) 10% fetal bovine serum, 0.8% sodium bicarbonate and L-glutamine[2] at 0.29 mg/mL.

VIRUS

The source of avian reoviruses WVU2937, 25, 59 and FC (4) and plaque titration methods in chick kidney tissue cultures have been described (4). Titers are expressed as plaque forming units (pfu) per mL. These strains represent four preliminary serotypes designated by Olson & Sahu (6).

ANTISERA

Antisera used are designated A to D as follows: A) Antiserum to strain WVU2937 was prepared as previously described (4). B) In order to follow development of antibody detected by the IFA

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[1]American Type Culture Collection, Rockville, Maryland.
[2]Grand Island Biological Co. of Canada, Burlington, Ontario.

method, 18 specific pathogen free (SPF)[3] chicks were inoculated with reovirus strain WVU2937 at three days of age (8). Chicks were housed together and serum was obtained from them when they were killed at intervals thereafter. Six control chicks housed in another isolation room were inoculated with uninfected tissue culture fluids and bled at corresponding intervals. C) Additional negative sera were obtained from 22 strictly isolated uninoculated SPF chickens at three to eight weeks of age. D) Eighteen unpaired sera were obtained from commercial broiler chicks at three weeks of age and again three weeks after oral inoculation with reovirus WVU2937 as part of another study.[4] These chicks were the progeny of breeders whose reovirus immune status was unknown.

CONJUGATED SERUM

Rabbit antichicken gamma globulin was purchased[5] and conjugated with fluorescein isothiocyanate (1). This conjugate was adsorbed with chicken liver powder (3) and tryptose phosphate broth (2) and stored in liquid nitrogen. The working dilution of the conjugate was determined by titration of the conjugate against dilutions of positive serum on reovirus infected cell monolayer cultures (described below). The highest dilution of conjugate reacting clearly with the highest dilution of positive serum was taken as the endpoint, and the working dilution was chosen as twice that concentration (3).

THE INDIRECT FLUORESCENT ANTIBODY METHOD

Monolayer Vero cells were trypsinized, suspended in growth medium at a concentration of 3×10^5 cells/mL and mixed with virus (strain WVU2937) suspension to give a ratio of cells to virus (pfu) of 3:1. Terasaki type[6] polystyrene plastic tissue culture plates (8 x 5.5 cm) containing 60 flat bottom conical wells, 3 mm diameter at the top and 1 mm diameter at the base, were each inoculated with 20 μL of the cell virus mixture, covered with loose fitting plastic lids and incubated at 37°C in a humid 5% CO_2 atmosphere. Uninfected cells were similarly seeded onto control plates.

Twenty-four hours later plates were fixed by a slight modification of the method described by Pursell and Cole (7). Six mL of phosphate buffered saline (PBS) pH 7.2 was added to each plate, followed by 24 mL of cold (-18°C) acetone added dropwise to give a final concentration of 80% acetone. After 30 min at room temperature the acetone was aspirated, plates were washed with PBS, blotted and then thoroughly air dried at room temperature in an isolation hood. Plates were then either used immediately or stored at -18°C overnight.

Serial twofold dilutions (commencing at 1:16) of heat inactivated (56°C/30 min) sera to be assayed for antibody were made in PBS and 10 μL of each dilution were added to each well. A similar volume of the first two dilutions (1:16 and 1:32) were added to the noninfected control monolayers. Plates were covered and incubated in a humidified incubator at 37°C for 30 min, then washed in PBS pH 7.2 at 4°C for 20 min and blotted but not thoroughly dried. Ten μL of the working dilution of the conjugate were then added to each well, plates were incubated at 37°C for 30 min, washed for 10 min each in two changes of PBS at 4°C, rinsed briefly in distilled water and blotted but not thoroughly dried.

Vero cells infected with reovirus strains 25, 59 and FC were also examined by the IFA method using antiserum to strain WVU2937.

MICROSCOPY

After the final blotting, plates were examined without further treatment, using a Zeiss photomicroscope II fluorescence microscope. The epi-fluorescence system incorporated an HBO-50 mercury vapor lamp, a BG-12 exciter filter, an FT510 chromatic splitter and an LP520 barrier filter. Plate wells were examined for fluorescence at a final magnification of 100 x using a 10 x achromat objective of numerical aperture (NA) 0.22 (Fig. 1). Higher magnification (160 x) was obtained if plates were inverted and examined with a 16 x neofluor objective, NA 0.4 and in this case clarity was improved if 10 μL of 10% glycerol-PBS solution pH 7.2 was added to each well.

Fluorescence was graded from 1+ to 4+ to indicate a range from faint to strong intensity and incidence. Only grades of 2+ or higher were recorded as positive. To determine if fluorescence could be related to the presence of virus particles in infected cells electron microscopy was conducted on Vero cell monolayer cultures infected as described for the IFA test, above.

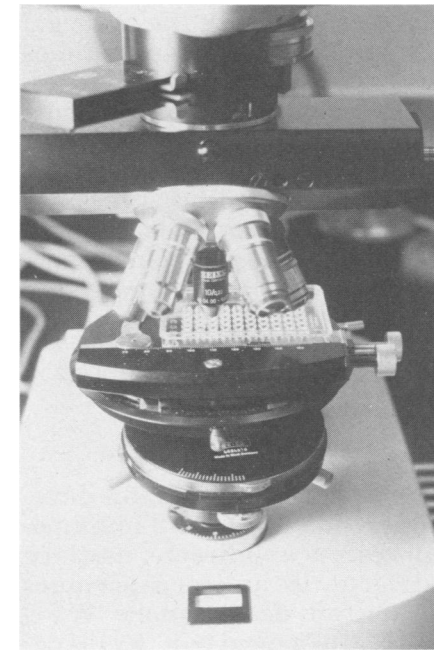

Fig. 1. Microculture plate on the mechanical stage mechanism of an epi-fluorescence microscope.

[3]SPAFAS Incorporated, Norwich, Connecticut.
[4]Sera were supplied by Dr. D. Key, Animal Pathology Laboratory, Sackville, New Brunswick.
[5]Cappel Laboratories, Cochranville, Pennsylvania.
[6]Grand Island Biological Co. of Canada, Burlington, Ontario.

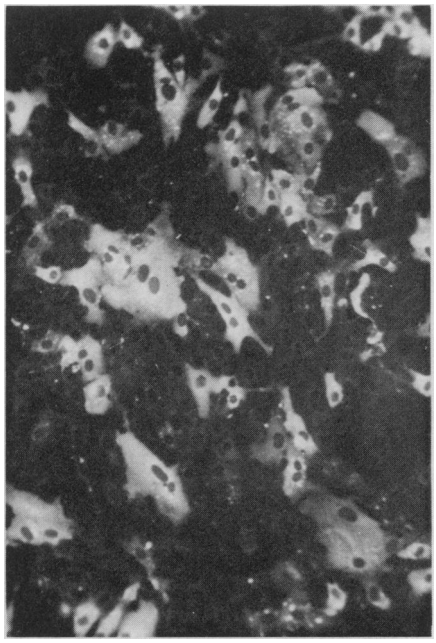

Fig. 2. Bright intracytoplasmic fluorescence was seen when reovirus infected Vero cells were examined 24 hours postinfection by the indirect fluorescent antibody method. X100.

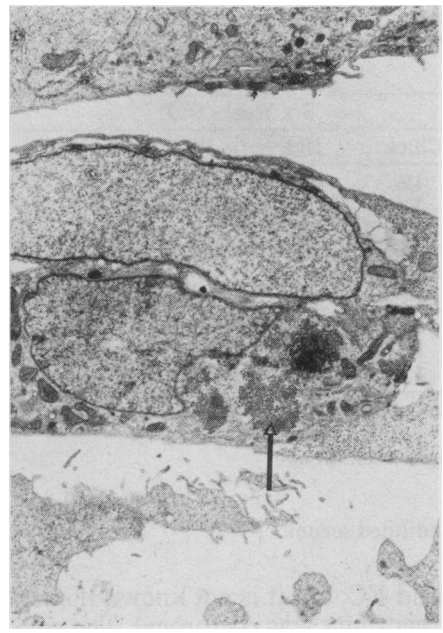

Fig. 3. Infected Vero cells exhibited cytoplasmic aggregates of virus particles (arrow) at 24 hours postinfection. X3730.

Twenty-four hours after infection cultures were fixed in glutaraldehyde and osmium tetroxide, embedded in Epon and stained with uranyl acetate according to standard methods (5).

RESULTS

Both infected and noninfected Vero cells formed a complete monolayer within 24 h after seeding onto the microculture plates. Although no cytopathic effect (cpe) was seen in infected cultures at this time, the indirect fluorescent antibody method using positive serum (serum A) revealed bright granular and diffuse intracytoplasmic fluorescence (Fig. 2) which progressively decreased in intensity and incidence (number of cells showing fluorescence) and eventually disappeared as the serum was diluted. Electron microscopic examination of duplicate cultures at the same stage of infection revealed virus aggregates in the cytoplasm (Fig. 3). No fluorescence was seen in negative control cultures overlaid with positive serum or in positive cultures overlaid with negative serum, prior to the addition of conjugate. Four replicate titrations of the positive serum in different passages of cells by the same person on different days gave end points of 1:128, 1:512, 1:128 and 1:256, respectively.

Reovirus infected experimental chicks (serum B) developed a progressive increase in IFA test titers with titers of up to 1:4096 detected by 30 days postinfection (dpi) (Table I). Only one of three chicks exhibited an IFA titer at 7 dpi. No sera were collected after 30 dpi and the persistence of titers was not determined. The 18 preinfection sera, sera from six uninfected control chicks and 22 other negative control chick sera of up to eight weeks of age were consistently negative. Comparative studies using different methods for antibody assay (Table II) indicated that IFA titers were lower than plaque reduction (PR) titers but in most cases the correlation between the two tests was positive. However, chicks giving negative results on the AGP test often gave positive results in IFA and PR tests, although three weeks after infection all three test methods gave positive results, with one exception (Table II).

DISCUSSION

Plastic microculture plates provided a rapid means of antibody assay by the IFA method. The size of the microculture plates allowed ease of manipulation on the microscope stage without modification of standard stage movement mechanisms. The small well size

TABLE I. Indirect Fluorescent Antibody Test Titers of Serum From Chicks Inoculated with Reovirus Strain WVU2937 at Three Days of Age

	Preinfection	Postinfection (day killed)					
Chick No.	1 day	1	7	10	18	21	30
1	—[a]	—					
2	—	—					
3	—		—				
4	—		—				
5	—		16[b]				
6	—			64			
7	—			256			
8	—				128		
9	—				128		
10	—					128	
11	—					—	
12	—					256	
13	—					256	
14	—					256	
15	—					256	
16	—					64	
17	—						1024
18	—						4096
Controls[c]	—	—	—	—	—	—	—

[a]Less than 16
[b]Reciprocal of serum dilution endpoint
[c]One control chick tested on each day indicated

TABLE II. Comparison of Indirect Fluorescent Antibody (IFA) Plaque Reduction (PRT) and Agar Gel Precipitation (AGP) Test Methods on Sera From Three and Six Week Old Commercial Broiler Chicks

Three Weeks Old[a]				Six Weeks Old[b]			
Chick	IFA	PRT	AGP	Chick	IFA	PRT	AGP
1	32[c]	—[d]	—[e]	10	—	256	—
2	64	256	—	11	64	512	+
3	64	1024	—	12	256	1024	+
4	128	1024	—	13	32	1024	+
5	—	1024	—	14	128	1024	+
6	—	—	—	15	256	1024	+
7	16	16	—	16	64	1024	+
8	32	256	—	17	512	1024	+
9	16	1024	—	18	1024	1024	+

[a]Not experimentally infected. Antibody detected presumed due to maternal immunity or natural infection
[b]Infected with reovirus strain WVU2937 at three weeks of age
[c]Recriprocal of dilution at endpoint
[d]Negative at 1:16
[e]Negative (—) or positive (+) AGP test using undiluted serum

ensured conservation of reagents and the small diameter of the well bottom, equal in area to the low power (100 x) microscope field, allowed rapid screening of a standard area without excessive scanning. Higher power magnification (160 x) was useful for detailed examination of fluorescent material but did not allow easy examination of the whole base of the well without additional manipulation and was not routinely used.

Fluorescence was seen equally well from either side of the plate under low (100 x) magnification. Thus, if laboratory contamination were considered to be a possibility, even after acetone fixation, plates could be sealed shut and examined from the reverse side.

Lack of fluorescence of control negative cultures overlaid with positive serum prior to exposure to the conjugate, negative results with preinfection sera and sera from negative control birds, and the progressive rise in the serum titers of infected birds attested to the specificity of the IFA test. Cross reactivity was demonstrated between antiserum to strain WVU2937 and virus strains 25, 59 and FC, but it is not known if such reactivity was reciprocal. The persistence of IFA reactivity of sera from positive birds has not been determined past 30 days after infection and the use of this test to detect long term reactors is not known.

The main disadvantage of the test is that a subjective interpretation is required for titer endpoint determination, and the reproducibility of serum titers determination by this method was up to a fourfold dilution range when assayed by the same person.

Titers detected by the IFA method were lower than those estimated by the PR test but were often positive when the AGP test was negative (Table II). Previous results (4) had shown that AGP titers did not persist as long as PR titers, and this was supported by these results, in which passive antibody in chicks was detected by the IFA and PR test methods but not by the AGP test. While comparisons have not been made between the IFA and ELISA methods it is anticipated that titers would be higher with the ELISA method (9).

The IFA method may be of use in laboratories requiring a rapid serological screening method in addition to or as an alternative to the ELISA method.

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